Animal Models of ischaemic stroke and characterisation of the ischaemic penumbra

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Abstract

Over the past forty years, animal models of focal cerebral ischaemia have allowed us to identify the critical cerebral blood flow thresholds responsible for irreversible cell death, electrical failure, inhibition of protein synthesis, energy depletion and thereby the lifespan of the potentially salvageable penumbra. They have allowed us to understand the intricate biochemical and molecular mechanisms within the 'ischaemic cascade' that initiate cell death in the first minutes, hours and days following stroke. Models of permanent, transient middle cerebral artery occlusion and embolic stroke have been developed each with advantages and limitations when trying to model the complex heterogeneous nature of stroke in humans. Yet despite these advances in understanding the pathophysiological mechanisms of stroke-induced cell death with numerous targets identified and drugs tested, a lack of translation to the clinic has hampered pre-clinical stroke research. With recent positive clinical trials of endovascular thrombectomy in acute ischaemic stroke the stroke community has been reinvigorated, opening up the potential for future translation of adjunctive treatments that can be given alongside thrombectomy/thrombolysis. This review discusses the major animal models of focal cerebral ischaemia highlighting their advantages and limitations. Acute imaging is crucial in longitudinal pre-clinical stroke studies in order to identify the influence of acute therapies on tissue salvage over time. Therefore, the methods of identifying potentially salvageable ischaemic penumbra are discussed.

Keywords: focal cerebral ischaemia, MRI, animal models, penumbra, cerebral blood flow

Abbreviations: CCA: common carotid artery, ECA: external carotid artery, ICA: Internal carotid artery, MCA: middle cerebral artery, MCAO: middle cerebral artery occlusion, CBF: cerebral blood flow, MRI: magnetic resonance imaging, DWI: diffusion weighted imaging, PI: perfusion imaging, SHRSP: spontaneously hypertensive stroke prone rat, LDF: laser Doppler flowmetry, OEF: oxygen extraction fraction, CMRO2: cerebral metabolic rate O2, CMRglu: cerebral metabolic rate of glucose consumption, ADC: apparent diffusion coefficient, MRS: magnetic resonance spectroscopy, ATP: Adeonosine triphosphate

Introduction

In the UK, stroke causes approximately 40,000 deaths per annum. It is the third leading cause of death and, importantly, the leading cause of long-term disability (Stroke Association: State of the Nation 2016). Restoration of cerebral blood flow (CBF) is the only proven effective treatment for acute ischaemic stroke. Tissue plasminogen activator (rt-PA), a thrombolytic drug, increases arterial reperfusion rates, restores perfusion and improves functional outcomes (Roth 2011). Mechanical thrombectomy has been shown to improve outcomes in the most severe cases of ischaemic stroke with proximal large artery occlusion, in numerous recent randomised trials (Berkhemer et al. 2015;Campbell et al. 2015;Goyal et al. 2015;Jovin et al. 2015;Saver et al. 2015). Despite these significant advances, there remains considerable need for alternative and adjunct treatments with less than 10% of acute ischaemic stroke patients receive intravenous thrombolysis and only a small proportion are eligible for thrombectomy(Chia et al. 2016;Henninger and Fisher 2016). In addition, most patients still have ongoing symptoms or disability after treatment and reperfusion itself can cause neuronal injury either through no-reflow or direct reperfusion induced injury to the tissue through hyperperfusion and haemorrhagic transformation (Nour et al. 2013). Despite successful recanalization with either thrombolysis or thrombectomy, incomplete reperfusion may still occur within the microcirculation (i.e capillaries, arterioles), a concept known as the 'no-reflow' phenomenon(Dalkara and Arsava 2012;Hauck et al. 2004), an effect which may be related to pericyte constriction and death in rigor (Hall et al. 2014). There is increasing evidence that this occurs in humans where restoration of tissue perfusion is a better predictor of outcome than recanalization alone(Soares et al. 2010). Therefore, therapeutic options that target the microcirculation following recanalization may act to improve outcome microvascular perfusion to the ischaemic territory and improve outcome. Therefore, animal models are still vital in understanding the pathophysiological mechanisms of ischaemic damage (e.g. collateral flow dynamics, microcirculation) and in developing and testing new treatments that could be given either as a stand alone treatment or in combination with thrombolysis/thrombectomy.

Animal models of focal cerebral ischaemia

There are a number of different models of focal cerebral ischaemia each with strengths and limitations when modelling the heterogeneous nature of clinical stroke. The majority of pre-clinical stroke studies are carried out in small animals with rodents (mice & rats) being the most common species used. Ischaemic stroke in humans most commonly occurs through an occlusion of the middle cerebral artery (MCA) and therefore MCA occlusion is the model employed in pre-clinical stroke studies. There has been a failure to translate neuroprotective strategies from the pre-clinical studies in animal models to the clinic which has questioned the validity of animal models of stroke. This translational roadblock has been the subject of many reviews and commentaries with both poorly designed pre-clinical studies and poor clinical trial design broadly to blame (Dirnagl and Macleod 2009;Howells et al. 2014). In 1999, a collective group of clinical and pre-clinical stroke experts met to try to understand and address this failure to translate. The Stroke Therapy and Academic Industry Roundtable (STAIR) group published their first set of guidelines for the improvement of pre-clinical studies in 1999 and have subsequently published further recommendations over the last 18 years (1999;Fisher et al. 2005;Fisher et al. 2009;Saver et al. 2009). Among the recommendations for pre-clinical stroke neuroprotection studies were the appropriate use of randomisation and blinding, use of co-morbid animal strains, using both male and female animals, investigation of appropriate dose response relationships and investigation in at least two independent laboratories. In light of the STAIR recommendations a number of meta-analyses have investigated study quality in pre-clinical stroke experiments. Many of these reviews and analyses have been quite damning for the pre-clinical stroke community demonstrating a lack of control of potential bias in stroke studies due to poor experimental design (i.e. lack of reporting of blinding, randomisation and insufficient power). Interestingly, this effect is not just within pre-clinical stroke research but across the biomedical field in general(Macleod et al. 2015). On a more positive note, the pre-clinical stroke community has also been at the forefront of improving quality of experimental studies. A number of scientific journals now require statements on power

calculations, randomisation & blinding in materials and methods and some editors are encouraging the submission of negative and neutral studies to address a recognised publication bias. There is still much work to be done, however, within the pre-clinical stroke community there is a strong commitment to move in the right direction. One example of this is the MULTIPART network which consists of pre-clinical and clinical stroke researchers as well as members involved in animal welfare and aims to provide a platform for international multi-centre preclinical trials in order to overcome the translational roadblock (http://www.dcn.ed.ac.uk/multipart/default.htm). The National Centre for the Replacement, Refinement and Reduction of animals in Research (NC3Rs) set up a working group (which included MULTIPART members as members from academia, pharmaceutical industry and UK Home Office) in 2014 aimed at improving the welfare of animals in stroke research as well as increasing the quality and rigour of experimental stroke research. They have recently published a set of guidelines aimed at improving in vivo stroke modelling and animal welfare(Percie du et al. 2017). A summary poster format of these guidelines are included as supplementary content to this review article.

Intraluminal filament model of MCAO

The intraluminal filament or suture model is by far the most commonly employed model of focal cerebral ischaemia. In order to induce middle cerebral artery occlusion (MCAO) an intraluminal filament or suture (silicon coated or heat blunted) is introduced into the internal carotid artery (ICA) and advanced until it occludes the origin of the MCA (Longa et al. 1989). This model therefore requires exposure of the carotid vessels on the neck and temporary occlusion of the common carotid artery (CCA) as well as the external carotid, occipital and pterygopalatine arteries in order to successfully advance the filament to occlude the origin of the MCA. Following insertion of the filament, it can be left in place permanently or withdrawn after a defined period of time to induce reperfusion of the MCA. One of the advantages of the filament model of MCAO is that it is less challenging technically than the diathermy models of MCAO learn the necessary surgical skills and

can be applied to both rat and mouse models, allowing modelling of both permanent and transient MCAO. There have been a number of refinements of the intraluminal filament model since its introduction, to improve the recovery of animals(Trueman et al. 2011). The choice of filament used to induce MCAO has been demonstrated to be important in reproducibility and mortality with more incomplete MCAOs and haemorrhage associated with heat blunted filaments when compared to silicon coated filaments(Tsuchiya et al. 2003). A recent study investigated the influence of a newer design of filament (bowling pin shaped tip) and compared this with three different conventional types of filament for recovery following permanent MCAO. The authors demonstrated that the bowling pin shaped filament reduced ischaemic damage (particularly in the hypothalamus & occipital region), reduced mortality and showed improved collateral filling from the posterior cerebral artery territory(Shanbhag et al. 2016). It is important for individual laboratories to carry out pilot studies in order to ensure the optimal size and type of filament for the particular strain and weight range of rats/mice used. This will ensure that successful occlusion is more likely thereby reducing variability and mortality from haemorrhage.

A recent study investigating two different surgical approaches for insertion of the filament in mice (via the CCA) or external carotid artery (ECA)) demonstrated an improved recovery of perfusion and survival when the filament was inserted through the ECA (Smith et al. 2015). However, the difference in mortality observed in this particular study may be related to patency of the CCA following reperfusion. When the authors inserted the filament through the ECA the CCA was kept patent. However, this was not the case when inserting the filament through the CCA where it was permanently tied off. Variations in the circle of Willis, particularly in mice, may result in variability in the extent of reperfusion following removal of the filament if the circle is incomplete and the ipsilateral CCA is permanently occluded. Other modifications in rats include maintaining patency of the external carotid vessels (ECA, occipital artery & pterygopalatine artery) in order to prevent damage to the facial muscles and palate therefore helping recovery of animals by minimising damage to the muscles of mastication and improving post-surgical weight loss and hydration.

Another refinement, with closed skull models such as the intraluminal filament model, that has been introduced to reduce mortality in the spontaneously hypertensive stroke prone (SHRSP) rat is to prepare a cranial burr hole prior to transient MCAO(Ord et al. 2012). We have more recently demonstrated that a cranial burr hole, made under the same anaesthetic used for inducing MCAO, is also associated with reduced mortality in the SHRSP (unpublished findings). This refinement could also be applied alongside reduced duration of ischaemia, when using co-morbid strains (i.e aged animals, obese, etc.) that may be associated with higher mortality following MCAO.

The intraluminal filament model of MCAO is commonly used for assessment of functional outcome after stroke allowing the impact of therapeutic interventions (i.e pharmacological, cell based treatments) to be determined. There are many different behavioural tests that are used with most of the commonly used tests assessing some level of sensorimotor function. With the filament model of MCAO there is typically damage to the striatum and depending on the length of occlusion time cortical damage will also be present. It is important that the behavioural test(s) chosen reflect the extent of damage in order to maximise sensitivity of detecting a change. In addition, neurological scores (i.e Bederson scale, modified neurological severity score) are often used as a quick method for assessment of neurological deficits however often lack sensitivity due to their subjective nature and recovery of rodents long term(Bederson et al. 1986;Garcia et al. 1995). There are a number of very good and comprehensive review articles on functional outcome after stroke in rodents (Schaar et al. 2010). One of the problems is a lack of consensus on the appropriate tests to be used with a large variation in behavioural tests being used across stroke models.

One of the limitations with the filament model of MCAO is the risk of partial (incomplete) occlusions of the MCA either due to; a) the filament size not being optimal for the diameter of blood vessel at the point of occlusion (addressed by indirect matching of filament to a pre-defined weight range); b) the filament has not been advanced far enough or has become dislodged following insertion. Often groups will use laser Doppler flowmetry (LDF) prior to, during and immediately following MCAO in

order to confirm a successful ischaemic insult and reperfusion. This involves the placement of a laser Doppler probe onto a single point on the skull (skull thinning at the probe location is recommended for rats because of the thicker skull) over the MCA territory. Successful occlusion of the MCA is determined by a reduction in CBF below a set threshold at a specified probe location. However, these thresholds can vary considerably from lab to lab. Positioning of the LDF probe is also crucial in order to ensure that it is placed over the ischaemic core territory. Due to its placement on the skull surface this means the signal is from the dorsal cortex which is typically where the ischaemic penumbra will be located due to the presence of the collateral vessels originating from the anterior cerebral artery. Therefore, depending on the strain and variability of lesion size, the percentage reduction of CBF may vary considerably due to the extent of collateralisation.   The predictive value of LDF for confirming MCAO and reperfusion is unclear and more data are required to determine whether a correlation exists between extent of LDF reduction and infarct size. Our own unpublished data demonstrate that LDF provides little if any predictive value for the size of the eventual infarct **(see Figure 1)**.

In terms of ischaemic penumbra, the intraluminal filament model has been demonstrated to produce a considerable volume of potentially salvageable penumbra with the presence of co-morbidities such as hypertension resulting in significantly less penumbra (McCabe et al. 2009;Meng et al. 2004;Shen et al. 2003). This makes it a useful model for studies investigating the impact of therapeutic approaches on either the volume or lifespan of the penumbra or tissue salvage following reperfusion(Henninger et al. 2007a;Henninger and Fisher 2007).

In order to overcome the issues with incomplete MCAO with the filament model, acute MRI scanning can be carried out immediately following insertion of the filament. Diffusion weighted imaging (DWI) will allow the extent and size of the early ischaemic damage to be visualised while MR angiography can confirm the absence of flow through the MCA and reestablishment following removal of filament. The benefits of carrying out baseline imaging for lesion volume during MCAO

means that one can use the same animal as its own control in order to assess the impact of reperfusion and/or treatment on this initial lesion, thereby eliminating some of the issues with variability. Our own laboratory has recently investigated the influence of early reperfusion in the SHRSP where we have observed that early reperfusion at 35 min can reduce baseline lesion volume thereby resulting in tissue salvage (**see Figure 2**). However, this is not always possible in stroke laboratories due to availability and cost issues surrounding MRI scanning.

One area of criticism levelled at the transient intraluminal filament MCAO model is that it does not represent the clinical stroke population where typically gradual recanalization of the occluded vessel will occur while in the animal model following filament removal there is prompt surge reperfusion (Hossmann 2012). Thrombolysis with rt-PA results in a gradual breakdown of the clot which can take anywhere from 30 mins to up to several hours to fully lyse and therefore may induce a gradual reperfusion (Alexandrov et al. 2001). In contrast with removal of the filament it will induce a surge of reperfusion which is unlikely to be observed with thrombolysis(Burrows et al. 2015). However, with the recent advent of endovascular thrombectomy demonstrating significant clinical efficacy in patients with large proximal clots, this has given the filament model a new found clinical relevance. Surge reperfusion observed with removal of the filament will be similar to that observed with endovascular thrombectomy (Sutherland et al. 2016).

Electrocoagulation model of MCAO

The electrocoagulation or diathermy model of MCAO in rodents was originally developed by Tamura and colleagues(Tamura et al. 1981). In this model a craniectomy is performed to expose the MCA on the brain surface. Electrocoagulation forceps are used to coagulate a particular portion of the MCA in order to permanently occlude the vessel. Following coagulation of the vessel some groups will then cut through the coagulated portion of the vessel to ensure complete occlusion. One of the advantages of this model is that the section of the MCA that is occluded can be varied (i.e. a distal or proximal occlusion) in order to induce a stroke affecting cortical or both cortical and sub-cortical

territory. This model has good reproducibility and typically less variability in lesion size in comparison to the filament model of MCAO. A distal occlusion of the MCA will induce a cortical lesion whereas a proximal occlusion (at the origin of the MCA), which includes the lenticulo-striate branches, will induce a larger sub-cortical and cortical infarct. Typically, mortality is low owing to the craniectomy required to visualise and occlude the MCA which limits the effects of oedema. One limitation with this particular model is that it induces permanent MCAO and therefore reperfusion is not possible. However, diathermy occlusion of the MCA can be replaced by use of a reversible ligature or clip but this also leads to greater variability in outcome (Buchan et al. 1992;Shigeno et al. 1985).   The model is also more technically challenging in terms of surgical skills necessary to carry out the craniectomy and expose the MCA without causing significant bleeding or damage to the underlying cortex. Due to the location of the craniectomy, damage to the temporalis muscle may occur particularly if a proximal occlusion is carried out and this can cause problems with recovery in terms of eating.   With this model we have demonstrated that there is a measureable region of penumbra using MRI PI/DWI mismatch, which gradually becomes incorporated into the infarct over the first 3-4 hours after MCAO (Tarr et al. 2013).

Embolic models of MCAO

In order to try to mimic the clinical situation, a number of models of thromboembolism have been developed for use in animal models. These models allow for the study of thrombolytic agents and their ability to break down the clot, recanalise the vessel and importantly, allow the potential of novel neuroprotective agents to be tested alongside thrombolysis. The most widely used thromboembolic model relies on the generation of an embolus for occlusion of the MCA. Emboli are pre-formed outside the body using autologous blood (Zhang et al. 2015). The clots, prepared in advance to a particular diameter and length, are loaded into a fine catheter which is introduced into the ICA and advanced to occlude the origin of the MCA in a similar surgical approach to the intraluminal filament model. However, the embolic model is associated with significantly higher

mortality and variability in lesion size (Macrae 2011). One issue is that the clot can break up and result in multifocal ischaemic lesions depending on where the fragments become lodged. Alternatively, the clot may travel further than intended and occlude a vessel other than the MCA such as the posterior cerebral artery. Advantages are that this model can be used to assess the impact of treatments with thrombolysis (rt-PA) or test new thrombolytics since recanalization does occur.

Photothrombosis models of MCAO

The Rose Bengal model of photothrombotic stroke was introduced in 1985. This model requires the intravenous injection of Rose Bengal (photosensitive dye) followed by the illumination of the skull by a laser in a specific cortical location. Illumination results in activation of the dye producing highly reactive oxygen radicals that induce endothelial damage, platelet activation and aggregation and ultimately the formation of thrombi(Watson et al. 1985). Advantages of this model are that it produces thrombi similar to the thrombi observed clinically, is relatively straightforward and quick to carry out the surgery necessary, and has lower variability since there is selective occlusion to the pial vessels around the illuminated zone. A recent study used a slightly modified version where they induced a proximal occlusion of the MCA with rose Bengal in mice producing a cortical and sub-cortical lesion. Interestingly, the authors demonstrated a sizeable volume of penumbra when assessed acutely with MRI (Qian et al. 2016). This opens up the possibility of assessing the impact of reperfusion with thrombolysis (i.e rt-PA) and/or neuroprotectants in a model, which displays a measureable penumbra.

Thromboembolic models of MCAO

In order to try to represent the clinical situation observed with rt-PA induced reperfusion, a mouse model of thromboembolic stroke was developed in 2007. In this model, a small craniotomy is made to expose the distal branches of the MCA on the cortical surface and thrombin is injected into the lumen of the blood vessel using a micropipette(Orset et al. 2007). The thrombin injection results in

the immediate formation of a fibrin clot at the site of injection thereby resulting in a rapid decrease in perfusion to the affected territory. The strengths of this model are in reproducibility, low mortality and suitability for testing thrombolytic drugs either alone or alongside adjunct therapies(Macrae 2011). It has limited value for generating data on neurological/sensorimotor deficits because of the small size and location of the infarct. Injection of rt-PA to induce thrombolysis results in the gradual breakdown of the clot taking around 30-50 minutes for the full restoration of perfusion (Orset et al. 2007). One other issue with this model is the development of spontaneous recanalization where Durand and colleagues demonstrated partial to complete reperfusion in 80% of animals at three hours after occlusion (Durand et al. 2012).

This model has been used by a number of groups since its development and a recent retrospective pooled analysis of the effectiveness of rt-PA (alteplase) in this model was carried out. The authors analysed data from 26 different studies from across 9 international centres and demonstrated that early administration of rt-PA (<3h) was associated with significant benefit. However, late administration (≥ 3h) had no, or a deleterious effect (Orset et al. 2016). This provides strong validation of this model being clinically relevant and therefore useful for the investigation of future neuroprotectants with thrombolysis and hope for successful translation. More extensive thrombotic MCAO is possible by topical application of ferric chloride (10-20% solution saturated on strip of filter paper) to the dura mater overlying the main trunk of the MCA (Karatas et al. 2011) or the common carotid artery followed by mechanically promoting embolization of FeCl3-triggered thrombi to the internal carotid artery (Martinez de et al. 2017). This model is particularly suited to in vivo real time studies of the cortex using laser speckle flowmetry or 2-photon microscopy.

Endothelin-1 model of MCAO

Endothelin-1 (ET-1) is a potent and long lasting vasoconstrictor peptide which makes it a valuable tool for inducing focal cerebral ischaemia. Originally developed in the rat, topical administration of ET-1 to the abluminal surface of the exposed MCA results in a significant and long-lasting

vasoconstriction with gradual reperfusion(Macrae et al. 1993). The model was subsequently modified for stereotaxic injection of ET-1 into tissue adjacent to the MCA(Sharkey and Butcher 1995) and this is by far the most common ET-1 method used in recent years. One advantage of this model over the abluminal administration is that the surgery is relatively quick and straightforward for targeting the MCA and avoids damage to the facial muscles. Another advantage is that a guide cannula can be implanted into the site in advance allowing ET-1 to be injected in conscious animals, thereby removing any confounds of anaesthesia(Moyanova et al. 1998). Disadvantages are a high variability in lesion volume that can occur due to variability in the response of the blood vessels to ET-1. Ansari and colleagues have tried to overcome this with the use of laser Doppler flowmetry during administration of ET-1 (Ansari et al. 2013) . It is unclear how much penumbral tissue this model produces. However, there are a number of studies that have used the model and demonstrated reductions in infarct volume with therapeutic approaches suggesting the potential for tissue salvage (Callaway et al. 2004;McCarthy et al. 2009;Stoop et al. 2017).

ET-1 can be stereotaxically injected into any region of brain parenchyma to induce a localised focal ischaemic lesion. By targeting specific neuroanatomical areas, such as white matter tracts, a discrete targeted anatomical lesion (e.g. internal capsule) can be induced to produce a specific behavioural deficit(Lecrux et al. 2008).

Ischaemic penumbra

The definition of penumbra is the region of potentially salvageable brain tissue which is by virtue a region of reduced blood supply in which energy metabolism is preserved (Hossmann 1994). The ischaemic penumbra was originally defined by Astrup and Symon in anaesthetised baboons based on CBF thresholds of viability (Astrup et al. 1977;Symon et al. 1977). They defined an upper CBF threshold of ischaemia of 20ml/100g/min (compared to a baseline value of 50-55ml/100g/min) where cells exhibited electrical failure, had sustained energy metabolism, low extracellular potassium, and a lower CBF threshold (CBF of 6ml/100g/min) where extracellular potassium was

increased alongside electrical failure and energy failure (Astrup et al. 1981). Tissue with CBF between these thresholds had the potential for recovery if perfusion was promptly restored. However, tissue with flow values below the lower threshold were destined for irreversible cell death. Our understanding of the penumbra has evolved over the subsequent years since these seminal experiments where we now understand considerably more about the possible lifespan of the penumbra following stroke. With the advent of acute imaging (PET & MRI) techniques, we can broadly summarise the ischaemic penumbra as the region of brain tissue that is hypoperfused, has maintained cerebral metabolic rate of oxygen consumption ($CMRO_2$) and an increased oxygen extraction fraction (OEF) (Marchal et al. 1996).

Ex-vivo autoradiographical techniques

With this in mind, the penumbra can be identified based on properties of CBF, energy metabolism (ATP, glucose metabolism), protein synthesis and tissue pH. These different components will cease to function at varying flow thresholds (see review by (Hossmann 1994). Protein synthesis inhibition occurs early after the onset of ischaemia due to endoplasmic reticulum stress and does not immediately cause irreversible cell death. The threshold for inhibition of protein synthesis occurs first (around 55ml/g/min or 50% reduction in rats) while energy metabolism is still ongoing at these flow values (Mies et al, 1991). The inhibition of cerebral protein synthesis (CPS) acutely following permanent MCAO (within 1 hour) has been shown to predict the final infarct size (Hata et al. 2000). CPS inhibition may be partially reversible and is not due to energy failure since ATP depletion is observed with more severe reductions in CBF. The mismatch between maintained ATP production and CPS inhibition has been used as a method for identification of the penumbra where the area of reduced CPS but maintained energy metabolism represents the penumbra (Hata et al. 1998;Hata et al. 2000). Cerebral metabolic rate of glucose consumption (CMRglu) can be obtained with ^{14}C-2-deoxyglucose autoradiography following MCAO allowing identification of regions of severely reduced, increased and normal CMRglu. We have previously used this technique to validate an MRI

technique (oxygen challenge) for identification of tissue metabolism (Robertson et al. 2011a). The metabolic penumbra has previously been identified as a region of increased CMRglu which shows moderate acidosis while the ischaemic core is the region of reduced CMRglu and severe acidosis(Peek et al. 1989). Based on tissue pH, Back and colleagues identified the ischaemic penumbra as a region of hypoperfused tissue with a region of acidosis and an alkaline sub-area while the core was severely acidic (Back et al. 2000).

These techniques have been crucial in developing our understanding of the different tissue compartments following stroke and identifying critical thresholds for energy metabolism, ion homeostasis and perfusion. They are however, limited to the research setting and do not allow for longitudinal assessment over time in the same animal since autoradiography is a terminal procedure. In order to longitudinally assess the evolution of ischaemic damage and lifespan of penumbra, the use of small animal MRI scanning has become invaluable. The advantages of pre-clinical MRI scanning is that these methods and protocols can be relatively easily translated to the clinical setting allowing development of new methods and assessment of the same outcomes to be compared.

MRI Perfusion-Diffusion mismatch

MRI Perfusion-Diffusion mismatch is currently used to provide an indirect assessment of the ischaemic penumbra in animal models and in acute stroke patients. The diffusion-perfusion mismatch can be used for patient recruitment into clinical trials ensuring only patients with remaining penumbral tissue are selected for the study of therapies and interventions designed to facilitate reperfusion. Pre-clinical stroke research employing diffusion and perfusion weighted imaging has increased considerably over the last decade with increasing access to small animal MRI scanners.

DWI measures the diffusion of water molecules in biological tissues and following ischaemic stroke changes in diffusion occur within minutes (Hjort et al. 2005;Norris et al. 1998). The apparent

diffusion coefficient (ADC) generated from DWI allows the magnitude of diffusion to be quantified. These diffusion changes are thought to be because of the pathophysiological processes associated with the initiation of the ischaemic cascade (i.e. bioenergetic failure, and failure of ion pumps) which result in cytotoxic oedema (cell swelling)(Sevick et al. 1992). The process of cell swelling results in a restricted diffusion of water molecules in the extracellular space and thereby a change in diffusion signal. This reduction in ADC occurs over the acute phase following stroke (minutes to days) followed by a normalisation and then increase in diffusion in the later phases due to the development of vasogenic oedema and cavitation(Shen et al. 2011). Therefore, early reductions in ADC reflect the development of ischaemic damage and have been shown to closely correlate with histopathological damage(Sevick et al. 1990). It was originally thought that the acute ADC lesion represented irreversibly damaged tissue but there is increasing evidence that at least some of the acute diffusion lesion has the potential for recovery depending on how quickly reperfusion is induced. Indeed, we have demonstrated this in the SHRSP rat (**Figure 2**) where early reperfusion can reverse some of the acute ADC lesion. Following permanent MCAO the DWI (or ADC) lesion will gradually evolve over the first few hours in animal models reflecting the incorporation of ischaemic penumbra into the ischaemic core. In healthy normotensive rats this has been shown to occur over the first 3-4 hours with the damage matching the final infarct volume at 24 hours post MCAO (McCabe et al. 2009;Meng et al. 2004). Longitudinal DWI imaging during the acute phase following MCAO can also be used as an indirect measure of penumbral volume by assessing the growth of the ADC lesion over time into the final infarct volume (Reid et al. 2012). This allows for the assessment of therapeutics or impact of risk factors/co-morbidities etc to be evaluated on the evolution of ischaemic damage. For instance, we have demonstrated the impact of hypertension, gender and acute post-stroke hyperglycaemia on the acute evolution of the ADC lesion and final infarct volume following permanent MCAO (Baskerville et al. 2016;Reid et al. 2012;Tarr et al. 2013). By understanding the acute evolution of ischaemic damage against a background of co-morbidities/risk factors etc. we can better understand the therapeutic time window with the potential of developing

stratified treatments for specific patient sub-groups that can slow down the infarct growth and prolong the time window for recanalization.

Perfusion imaging (PI) provides information about the perfusion status of the brain. This can be carried out using contrast enhanced techniques or by using blood as an endogenous contrast agent with arterial spin labelling (ASL) methods. Following MCAO, PI can be used to assess the spatial extent of CBF reduction using specific CBF thresholds. These CBF thresholds may vary depending on the method used to carry out PI as well as species, strain and scanner (see review by (Campbell and Macrae 2015)). Following permanent MCAO the perfusion deficit remains relatively constant during the first hours post- MCAO. This region of haemodynamic compromise shown on PI is typically larger than the region of DWI or ADC abnormality, resulting in the so called 'perfusion diffusion mismatch'. This mismatch tissue comprises tissue that is hypoperfused (perfusion deficit) but does not show signs of cytotoxic oedema (DWI or ADC lesion) **(Figure 3)**. Perfusion diffusion mismatch predicts the tissue that will further evolve into the diffusion abnormality and become irreversibly damaged if reperfusion or acute neuroprotection is not initiated(Warach 2003). Therefore, this approximates the potentially salvageable ischaemic penumbra.

One of the advantages of this technique in animal models is that the acute evolution of ischaemic damage can be longitudinally assessed in the same animal. A number of studies have investigated the evolution of the PI/DWI mismatch following permanent MCAO in rats and have demonstrated that the mismatch gradually disappears over the first 3-6 hours consistent with the loss of penumbra and enlargement of the ischaemic core (DWI lesion). This allows for the dynamic nature of ischaemic damage, CBF and loss of penumbra to be assessed either with/without a therapeutic intervention(Henninger et al. 2007b) or for the impact of stroke co-morbidities on acute evolution to be determined (Reid et al. 2012;Tarr et al. 2013). In addition, acute scanning can be carried out during MCAO (i.e. DWI and PI) with reperfusion then induced remotely within the scanner either by filament withdrawal or intravenous administration of rt-PA in embolic models. Further, the impact

of reperfusion on the acute evolution of lesion volume and extent of reperfusion can be assessed alongside investigation of the fate of penumbral tissue following reperfusion.

Metabolic MRI techniques

One of the limitations of the PI/DWI mismatch technique is that penumbra is not defined on metabolic status or OEF. Since the technique is dependent on the setting of imprecise thresholds, a portion of the diffusion lesion may contain penumbral tissue while the perfusion lesion may contain benign oligaemic tissue which is not at risk of infarction. Several studies including our own (**Figure 2**) have demonstrated that the early DWI abnormality is partially reversible upon reperfusion, demonstrating that this may indeed include penumbra(Labeyrie et al. 2012;Li et al. 2000). Therefore, there has been considerable effort to develop more accurate MRI techniques that can differentiate tissue based on metabolism. Using a T_2^* weighted MRI sequence, most commonly used in functional MRI studies, Santosh and colleagues(Santosh et al. 2008) used an 'oxygen challenge' to exploit the paramagnetic properties of deoxyhaemoglobin and the difference in deoxy/oxyhaemoglobin ratio in the penumbra compared to surrounding tissue. This technique has been extensively validated with histology, glucose metabolism and reperfusion (Robertson et al. 2011a;Robertson et al. 2011b;Robertson et al. 2015) and by other groups (Shen et al. 2015). Further development has included the use of oxygen carriers (perflourocarbons) in order to amplify the signal change within the metabolically active penumbra (Deuchar et al. 2013).

Following arterial occlusion, one of the immediate consequences is energy failure due to insufficient delivery of substrates for glucose metabolism. This results in a switch from aerobic to anaerobic glycolysis thereby increasing lactate levels. A number of magnetic resonance spectroscopy (MRS) studies have demonstrated changes in tissue lactate levels following experimental stroke. A recent study carried out lactate MRI and lactate spectroscopy to characterise the ischaemic penumbra based on the change of lactate levels following a challenge with 100% O_2 (Holmes et al. 2012a). In this particular study, lactate levels were elevated within the ischaemic hemisphere acutely following

permanent MCAO (within the first 3 hours) indicative of the presence of anaerobic glycolysis. Ventilation of rats 100% O_2 in place of air during MRI scanning resulted in a decrease in lactate levels within the PWI/DWI mismatch region while no change in lactate was observed in the ischaemic core. This suggests that lactate imaging and spectroscopy alongside an O_2 challenge has the ability to identify tissue capable of recovery based on the ability to switch from anaerobic to aerobic glycolysis and *vice versa*(Holmes et al. 2012b). The advantage of these more recent techniques is that they probe tissue integrity based on metabolic activity and therefore may be able to identify patients with potentially salvageable tissue outside the current therapeutic time window.

Conclusions

There are a number of different animal models of focal cerebral ischaemia each with strengths and limitations. The choice of appropriate model depends on the research questions to be addressed with no models mimicking the heterogenous nature of stroke in humans completely. For instance, intraluminal filament induced transient MCAO would be useful for investigating adjunctive treatments that could be given alongside endovascular thrombectomy while models of permanent MCAO could be used to evaluate neuroprotection therapies in the absence of reperfusion. In order to determine the therapeutic efficacy of novel neuroprotectants (with and without reperfusion) the use of longitudinal imaging (prior to and post treatment) provides greater insight into the mechanisms (i.e. perfusion, penumbra), therapeutic time-window and statistical power by being able to use each animal as its own control. The use of animal models that incorporate factors known to influence stroke outcome (i.e. hypertension, diabetes, ageing, gender) should also be used. However, an appreciation of the impact of risk factors on penumbra and outcome are essential in order to determine the optimal time window for administration of treatment and reperfusion.

Figure legends

Figure 1. Lack of correlation between the % reduction in laser Doppler flow signal (LDF) following insertion of the filament and infarct volume. LDF was measured at baseline and throughout MCAO and reperfusion. Infarct volume was measured by T_2 MRI at 72 hours following 90 min transient MCAO. Linear regression demonstrated no correlation between drop in LDF following MCAO and infarct volume (Pearson correlation).

Figure 2. A. Representative ADC maps of acute diffusion lesion at 30 min during a 35 min MCAO and T_2-weighted final infarct volume at day 7 in the SHRSP. B. Individual animal data for acute lesion volume during MCAO and final infarct volume at day 7 following reperfusion.

Figure 3. PI/DWI imaging at 1 hour following permanent MCAO (intraluminal filament) in a male Sprague Dawley rat. A. CBF map showing the perfusion deficit; B. ADC map showing the region of reduced diffusion (ischaemic damage); C. Thresholded perfusion and diffusion maps showing the mismatch (highlighted in green).

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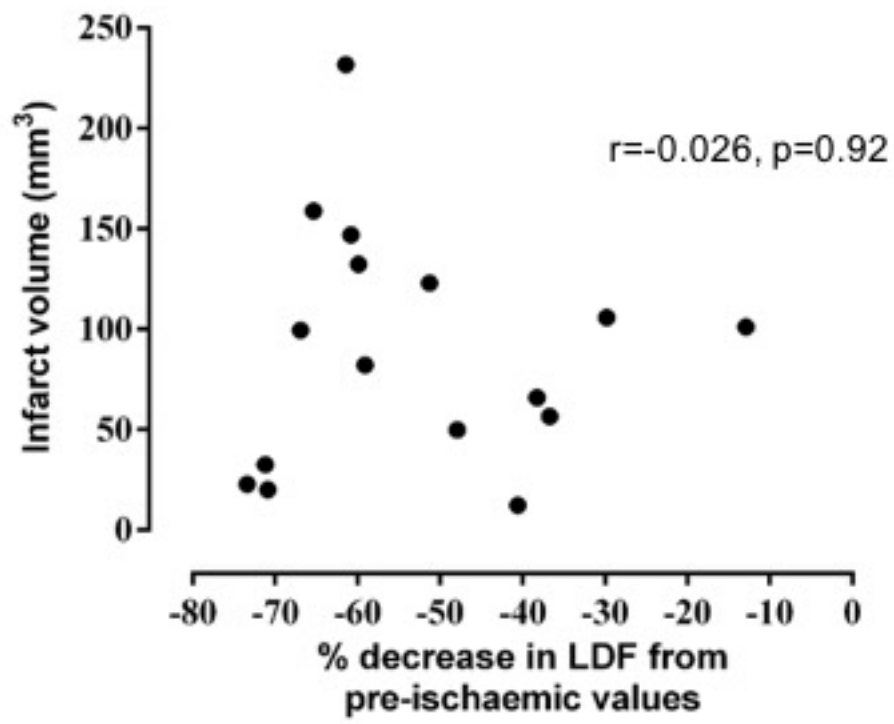
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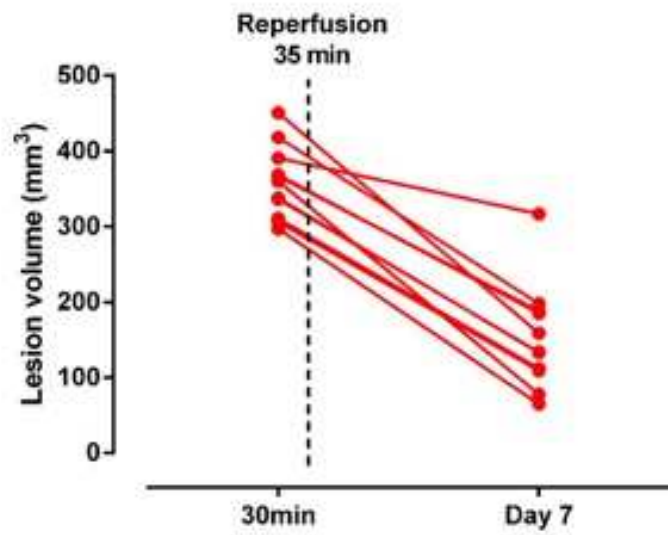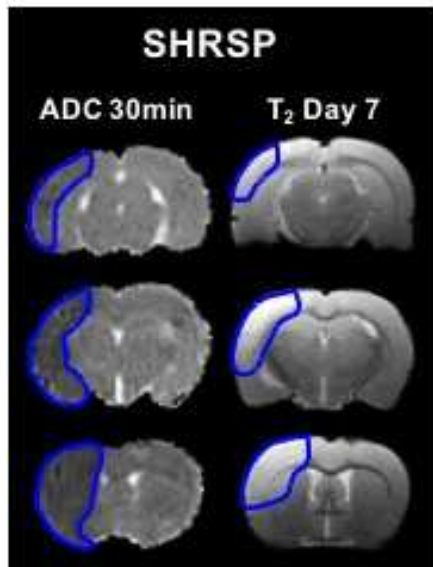
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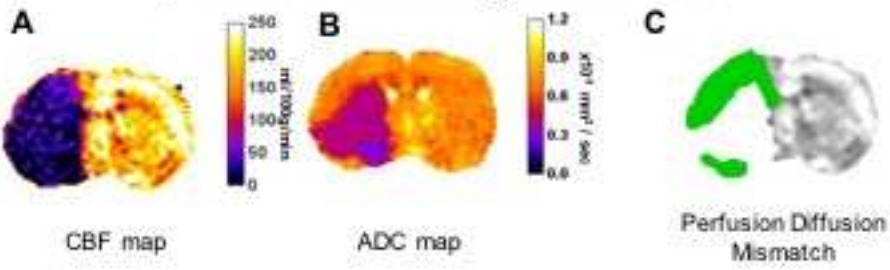
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r=-0.026, p=0.92

SHRSP

ADC 30min   T₂ Day 7

Reperfusion
35 min

PI/DWI mismatch following MCAO in the rat

A

250
200
150
100
50
0

ml/100g/min

CBF map

B

1.2
0.9
0.6
0.3
0.0

x10⁻³ mm² / sec

ADC map

C

Perfusion Diffusion
Mismatch

Highlights

- Discussion of the main animal models used for experimental stroke
- Importance of the ischaemic penumbra
- Acute imaging techniques for identification of the ischaemic penumbra